changes in relative orientations between the two receptor molecules from the transient complex to the 1:2 native complex are similar for the three cytokine-receptor systems. We thus propose a common model for receptor activation by class I cytokines, involving combined scissor-like rotation and self-rotation of the two receptor molecules. Both types of rotations seem essential: the scissor-like rotation separates the intracellular domains of the two receptor molecules to make room for the associated Janus kinase molecules, while the self-rotation allows them to orient properly for transphosphorylation. This activation model explains a number of experimental observations. The transient-complex based approach presented here may provide a strategy for designing antagonists and be useful for elucidating activation mechanisms of other receptors.

3377-Pos Board B238

Diffusion of Single B Cell Receptors in Resting and Stimulated B Lymphocytes using Super-Resolution Microscopy

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The B cell antigen receptor (BCR) is an integral part of our immune systems that communicates binding of antigen in the extracellular environment through the plasma membrane to the cytoplasm. Antigen binding to the BCR results in phosphorylation of intracellular tyrosine activating motifs (ITAMs), which act as binding partners for SH2 domain containing adapters and Src family kinases. To examine the spatio-temporal dynamics of the BCR during stimulation, we utilize stochastic optical reconstruction microscopy (STORM) in live CH27 cells. Using Alexa-647 labeled anti-IgM Fab fragments, we resolve the BCR down to tens of nanometers with acquisition rates of 40 frames per second. We then examine BCR diffusion by tracking the localized receptors and found that the BCRs in resting cells exhibit a lognormal distribution of diffusion constants centered at 10-1 μm2/s. Upon stimulation, this population is quickly shifted to one centered at 10-2 μm2/s. In separate experiments, we examined the calcium response of CH27 B cells and found that the buffers used for STORM experiments do not alter calcium responses after receptor crosslinking. We also perform an auto correlation analysis of the localized receptors, which shows an increase in BCR clustering on the same timescale as the reduction in diffusion. In addition, we are investigating the dynamics of other proteins involved in BCR signaling, namely the Src family kinase Lyn and Ezrin. We use two color simultaneous emission of a photoswitchable fluorescent protein mEos2 in conjunction with Alexa-647 in fixed and live cells to resolve the behavior of these interaction partners with respect to the BCR.

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Rho Family Proteins in High- and Low-Affinity fMLF Receptors Signaling in Neutrophils

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Bacterial or mitochondrial peptide N-formyl-MLF (fMLF) binding with specific receptor activates chemotaxis, secretory degranulation and oxidative burst of the neutrophils. High and low affinity fMLF receptors (FPRs) can initiate different patterns of signaling components (Fu et al., 2004; Safronova, Gabdoulkhakova, 2009). Main functions of Rho-family proteins are signal transduction from various membrane receptors and regulation of the cytoskeleton. We suppose that small G-proteins are the node of FPRs signal transduction and divergence. Aim of the work was to find out the role of Rho family small G-proteins in signaling from high- and low-affinity receptors to NADPH oxidase. The study was carried out on the induced peritoneal neutrophils of Balb/c male mice. The immunofluorescent staining with specific Abs against RhoA and Rac1/2 was used to visualize the localization of Rho-proteins in the cells. Rho-proteins are evenly distributed in cytoplasm of non-stimulated cells. Translocation of Rho-GTPases to membrane occurs under the stimulation of neutrophils with fMLF that indicates their activation. High dose of fMLF (50 mkM) induces brighter fluorescence of juxtamembrane area in comparison with low dose (1 mkM). Formylated peptide fMLF stimulates dose-dependent activation of the neutrophil respiratory response. Rho activator reduces intensity of the respiratory burst under the stimulation of high-affinity receptors, but does not influence the respiratory burst initiated by low-affinity FPRs. Rac/Cdc42 activator also down-regulates the respiratory response to low concentration of fMLF without effect in case of high dose. Thus, Rho-family small GTPases participate in signal transduction from the FPRs and reduce activity of NADPH-oxidase.in the stimulation of the high-affinity FPRs. This effect of Rho-family proteins can endow the reciprocal relationships between chemotaxis and oxidative activity in process of the neutrophil movement within the gradient of the chemoattractants.

3379-Pos Board B240

Activation of the Innate Immune Receptor Dectin-1 by Clustering

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Dectin-1 is a pattern-recognition receptor that plays a key role in anti-fungal immunity. It specifically recognizes beta-glucans, polymers of glucose found on the fungal cell wall. Upon ligand binding, dectin-1 triggers signaling cascades that lead to various cellular responses, including phagocytosis and the release of pro-inflammatory mediators, which all culminate in the generation of an effective immune response and eradication of the pathogen. The molecular mechanisms underlying dectin-1 activation and signal transduction are not fully understood. The aim of this study is to elucidate such mechanisms. We propose that upon binding of fungal particles, dectin-1 receptors cluster and assemble into multimeric complexes. In these clusters, dectin-1 becomes activated and promotes the recruitment and activation of Syk (spleen tyrosine kinase), which in turn triggers subsequent signaling cascades. To test our hypothesis, we stably expressed human dectin-1 in RAW 264.7 macrophages. Various levels of dectin-1 clustering were induced using antibody cross-linking or beta-glucans of different molecular weights. The corresponding stimulation of dectin-1 was detected by determining the activation of several downstream effectors (Src, Syk, NF-kB). We demonstrate that antibody cross-linking and larger ligands are able to induce more Syk phosphorylation than smaller ligands. To quantify the level of dectin-1 clustering, single molecule analysis was employed. Upon antibody cross-linking or ligand binding dectin-1 formed nanodomains of about 500 nm of diameter, which were identified as the nucleation site for intracellular signaling. Additionally, the phosphorylation and recruitment of Syk to regions of the plasma membrane rich in dectin-1 clusters was observed using confocal microscopy. Together, our results suggest that receptor clustering, is the mechanism by which dectin-1 is activated.

3380-Pos Board B241

Signal Processing by the Control Cycle of the IKK Kinase in the NFκB Signaling Axis

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The inflammatory response transcription factor NFkB, a key component of the immune system, shows intricate stimulus-specific temporal dynamics. It has been proposed that those dynamics play a role controlling the genetic outcome of inflammatory signals and hence the specificity of the cellular response to cytokines and other stimuli. As malfunctions in NFkB signaling are linked to many immune diseases as well as the onset and development of cancers, it is of considerable clinical interest to understand the mechanisms that control NFkB dynamics. Here we focus on IKK, a hub kinase that targets the main negative regulators of NFκB activity and onto which diverse receptor associated signals converge. Biochemical evidence suggests that IKK is regulated via a multi-state regulatory cycle and therefore we hypothesize that it can operate as a modulator, actively reshaping the signals generated at the receptor proximal level. Here we demonstrate that the IKK control cycle can function in at least three dynamical regimes some of them producing signals comprising multiple temporal phases with distinct coding capabilities. In particular, we show that the simplest three-state regulatory cycle generates biphasic signals with an early phase well suited for relaying information about stimuli amplitude and a late phase more apt for encoding stimulus duration. This study demonstrates that an actively regulated hub kinase can play a crucial role functioning as a signal ''categorizer'' classifying complex incoming signals into a limited set of output activities. Expanding the general analysis to a more detailed model of IKK regulation revealed how specific features of IKK and NFkB activities are controlled by the different enzymatic mechanisms within the kinase cycle.

3381-Pos Board B242

Integrating Single Molecule Techniques to Investigate Antigen-Independent Effects of IgE Binding to FcεRI

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Using single particle tracking and super-resolution, we have examined the influence of IgE binding on FcεRI mobility and distribution. The high affinity IgE receptor, FcεRI, of mast cells is an abγ2 tetramer, where the a subunit binds IgE and the b and γ subunits contribute ITAM-motifs for signaling. IgE binding is known to stabilize the receptor on the cell surface and recent evidence has shown that IgE plays a role in cell survival. Furthermore, the cytokinergic class of IgEs can induce signaling in the absence of antigen-mediated crosslinking. To examine IgE- and antigen-independent receptor behavior, we have generated a Fluorogen Activating Peptide (FAP)-tagged γ-subunit that allows us to track the non-IgE-bound receptor. Fluorogen activating peptides (FAPs) are genetically expressible tags that increase the fluorescence excitation cross-section of dye binding partners by up to four orders of magnitude, with bound lifetimes of up to ten seconds. These properties make the FAP system a convenient expressible probe for single particle tracking. We found that binding of non-cytokinergic IgE did not alter FcεRI mobility. However, in the presence of cytokinergic IgE (SPE7), we observed a decrease in mobility that is dependent upon accessibility of the SPE7 variable region. The far red emission of the malachite green based fluorogen (MG2p) is also advantageous for simultaneous tracking of FAP-γ with respect to GFP-tagged proteins, such as clathrin, to identify regions of receptor localization. To identify changes in receptor distribution associated with binding of cytokinergic IgEs, we use multi-colored direct STORM to simultaneously image the receptor bound to cytokinergic and non-cytokinergic IgEs, and compare this to the receptor distribution in the absence of IgE.

3382-Pos Board B243

Super-Resolution Tracking and Imaging of IgE Receptors during Stimulation by Antigen

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In mast cells, cross-linking of IgE bound to FcεR1 receptors with multivalent antigen initiates cell activation and consequent inflammatory responses. Clustered IgE-FcεRI interacts with early signaling partners such as Lyn, Syk, and LAT, initiating a signaling cascade. Our previous scanning electron microscopy (SEM) studies quantified the nanoscale co-redistribution of FcεRI with Lyn, Syk, and LAT within the plasma membrane upon antigen stimulation. Here we use super-resolution localization microscopy to localize proteins on the cell surface with sub-diffraction resolution using a TIR fluorescence microscope. We present, in snapshots, the time-dependence of IgE receptor reorganization after antigen addition using chemically fixed cells and quantify the extent of clustering using pair autocorrelation functions. We have validated our methods for quantifying and correcting for artifactual clustering due to probe over-counting through two-color experiments where individual IgE proteins are labeled with one of two species of distinguishable fluorescent probe and imaged at sub-diffraction resolution in multiple color channels. Here clustering is quantified using pair cross-correlation functions. Results of one- and two-color super-resolution measurements agree well with conclusions from SEM experiments. We have also extended this technique to imaging living cells at room temperature to measure IgE receptor redistribution in real time. We quantify the organization and diffusion behavior of proteins undergoing stimulated responses with the excellent statistics afforded by the technique. Live cell experiments are conducted under a variety of stimulation conditions, such as in the presence of drugs that perturb membrane physical state or affect actin cytoskeleton assembly. Our results confirm that super-resolution imaging is an effective tool for quantitative imaging of cellular components at the nanoscale. Our ongoing studies are providing new information to clarify the physical basis for spatial assembly of specific proteins in the plasma membrane during early signaling events.

3383-Pos Board B244

Somatostatin and Neuropeptide-Y Differentially Inhibit Insulin Secretion

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Glucose homeostasis is a tightly regulated process coordinated by insulin and glucagon secretion. In β-cells, G-protein coupled receptor (GPCR) activation facilitates the optimization of insulin secretion. Somatostatin (SST) and Neuropeptide-Y (NPY) inhibit insulin secretion through Gi activation; this inhibitory effect is mediated by the SSTR2/5 and Y1 receptors, respectively. Here, we questioned the mechanism by which SST and NPY attenuate insulin secretion. Two-photon excitation microscopy was utilized to measure the combined autofluorescence of NADH and NADPH (NAD(P)H, a cellular redox state indicator) upon SST or NPY application. Treatment with SST or NPY induces a supplementary increase in NAD(P)H autofluorescence at glucose levels ranging from 5-23 mM compared to untreated control, suggesting that the inhibitory effect of SST and NPY is not mediated through metabolic inhibition. We then tested the effect of these GPCR ligands on intracellular calcium ([Ca2+]i). At glucose concentrations above ~7 mM, pancreatic islets display synchronous oscillations in [Ca2+]i, resulting in the pulsatile release of insulin. SST treatment decreases the [Ca2+]i oscillation frequency, while NPY has no effect; the oscillation amplitude is not altered in the presence of either ligand. Insulin secretion assays were performed at low (2.8 mM) and high (16.7 mM) glucose levels to determine the effect of SST and NPY on the Gi pathway. At both glucose concentrations, mSIRK, a Gβγ-activating peptide, significantly potentiates insulin secretion; treatment with mSIRK(L9A), which serves as a negative control for mSIRK, does not affect secretion. However, combination treatment with SST and mSIRK inhibits insulin secretion at high glucose compared to mSIRK alone. Insulin secretion is not altered with NPY and mSIRK treatment compared to mSIRK only. These data suggest that SST attenuates insulin secretion through inhibition of Gβγ acting on voltage-gated Ca2+ channels and/or phospholipase-C, while NPY inhibits insulin secretion through Gα.

3384-Pos Board B245

Revealing Fibroblast Growth Factor Receptor-1 and Klotho-Beta Plasma Membrane Dynamics with FRAP and Number and Brightness Analysis

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Fibroblast growth factor-21 (FGF21) is a newly appreciated endocrine ligand that signals through fibroblast growth factor receptor-1 (FGFR1). Long term FGF21 treatment has been shown to decrease blood glucose levels in diabetic mouse models. Consequently, the signalling pathway of FGF21/FGFR1 has become a target for the management of diabetes. FGF21 activity requires the expression of a unique co-receptor Klotho-beta (KLB). This dependence suggests FGF21 signals through a FGFR1/KLB complex. To investigate the molecular interactions between FGFR1 and KLB at the plasma membrane, we have created fluorescently-labelled constructs of these receptors. Through FRAP, we revealed that KLB has a significantly shorter recovery half-life than FGFR1. After the addition of lactose, a competitive inhibitor of galectin lattice binding, the recovery half-life of KLB increased with no effect on FGFR1. After addition of FGF21, cells co-expressing KLB and FGFR1 also showed increased recovery half-life. This suggests that KLB interacts with the galectin lattice and co-expression of FGFR1 removes this interaction. To study complex formation of FGFR1 and KLB with the addition of FGF21, we employed the recently developed technique of Number and Brightness Analysis. It was found that KLB oligomerizes greater than 2-fold with the addition of FGF21; however, treatment with lactose abolished this response. In contrast, FGFR1-expressing cells showed no change in oligomerization state. In cells co-expressing KLB and FGFR1, FGF21 induced no significant change in KLB aggregation state while inducing a nearly 2-fold increase in FGFR1. Overall, these data suggest KLB associates with the galectin lattice in the absence of FGFR1, and that FGFR1 and KLB form a 1:2 complex that transitions to 2:2 upon addition of FGF21. Future studies will address how these interactions affect the downstream signalling of this important endocrine factor.

3385-Pos Board B246

Analysis of EGFR Signaling in Single Cells using Capillary Electrophoresis

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The importance of protein kinases in cell signaling and human disease is well established. While much is understood about how these enzymes regulate the cellular environment, the direct measurement of kinase activity in single intact cells remains challenging. In the present work, a novel assay for measuring Epidermal Growth Factor Receptor (EGFR) activity in single cells has been developed. EGFR is a receptor tyrosine kinase known to play a role in numerous cancers and is implicated in the inflammatory response to air pollution. The analysis of EGFR activity in clinical samples would provide valuable information regarding mechanisms of disease and potential interventions, however these samples tend to be small, heterogeneous mixtures of primary cells that are difficult to analyze using existing technologies that require large cell populations (e.g. Western blotting, flow cytometry) or expression of a genetically encoded sensor (e.g. FRET). We have developed an assay for measuring the phosphorylation dynamics within single intact cells using fluorescent substrate peptides in conjunction with capillary electrophoresis (CE). A peptide reporter is loaded into the cell of interest, phosphorylated by the intact cellular machinery, and then the cell is lysed and its contents analyzed by CE using high sensitivity laser-induced fluorescence detection. The ability to select individual cells coupled with sensitivity to 10^{-21} mol allows effective analysis of the clinical samples we are interested in studying. This technology has been validated using purified EGFR kinase domain and cell lysates, where the reporter is resistant to degradation for at least 1 hour. Additionally, phosphorylation (16.9% in 90 seconds) has been observed in an intact A431 cell treated with the phosphatase inhibitor sodium pervanadate. Single cell studies are ongoing